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Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms

JONGEJAN (F.), VOGEL (S.W.), GUEYE (A.), UILENBERG (G.). Vaccination contre la cowdriose avec des *Cowdria ruminantium* atténuées *in vitro*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 223-227

Des passages successifs de *Cowdria ruminantium* (stock Sénégal) dans des cultures de cellules endothéliales ombilicales bovines ont produit une perte de virulence sans perte d'immunogénicité, comme il a été démontré antérieurement. Dans une nouvelle expérience, 39 moutons néerlandais ont été immunisés avec des rickettsies atténuées du 21e passage et ont été éprouvés avec le stock homologue et des stocks hétérologues de *C. ruminantium*. Suite à l'immunisation, plusieurs des moutons ont montré une hyperthermie pendant 2 jours au plus, sans présenter une autre réaction clinique à la vaccination. Tous les moutons ont développé des titres élevés d'anticorps contre *Cowdria*. L'épreuve homologue virulente de 10 moutons n'a provoqué aucune réaction clinique, démontrant ainsi une immunité solide. Les réactions aux épreuves hétérologues ont varié entre la presque absence de réaction et la cowdriose mortelle, selon le stock utilisé. Les résultats sont commentés par rapport aux méthodes d'immunisation contre la cowdriose qui existent actuellement. Au Sénégal, 30 moutons sahéliens sensibles ont été immunisés avec des rickettsies atténuées du passage 21. Treize d'entre eux ont présenté une hyperthermie, le seul autre symptôme clinique fut une diarrhée passagère. Les animaux immunisés sont à présent exposés à l'infection naturelle dans les Niayes, région d'où le stock Sénégal a été isolé à l'origine.

Mots clés : Ovin - Cowdriose - *Cowdria ruminantium* - Culture de cellule - Cellule endothéliale bovine - Technique immunologique - Vaccin atténué - Anticorps - Vaccination - Virulence - Hyperthermie.

INTRODUCTION

Protective immunity against cowdriosis can be induced in susceptible ruminants by infection with virulent blood and subsequent treatment of the reaction with antibiotics (1). This type of immunization is practiced in South Africa using virulent sheep blood infected with the Ball 3 stock, followed by treatment with tetracyclines (13). Although useful to control the disease, the vaccine is far from ideal. In addition, the existence of distinct antigenic differences between *Cowdria* isolates (4, 5, 9, 10) may explain the occurrence of clinical cowdriosis in animals that were vaccinated with this vaccine based on the Ball 3 isolate.

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Sequential passage of *Cowdria* in bovine endothelial cell (BUE) cultures and the resulting attenuation of a Senegalese isolate of *Cowdria* has been reported recently (12). We have carried out further immunizations of sheep with the attenuated *Cowdria* vaccine. Here we report on the response of European and African sheep to vaccination and of European sheep to homologous and heterologous *Cowdria* challenge under laboratory conditions.

MATERIAL AND METHODS

Cowdria stocks

Seven stocks of *Cowdria ruminantium* were used : a Senegalese isolate, designated "Senegal" (9), two South African stocks, "Welgevonden" (3) and "Ball 3" (7), an isolate from Guadeloupe, "Gardel" (15) and three other stocks from Africa, "Um Banein" from the Sudan (8), "Umpala" isolated by M. ASSELBERGS in Mozambique (unpublished) and the "Lutale" stock from Zambia (9).

All stocks were stored as infective blood stabilates in liquid nitrogen with DMSO as cryoprotectant. The infectivity of the isolates had been tested before in susceptible goats (Saanen breed) by intravenous inoculation of 2 ml aliquots of thawed blood stabilate (9, 10, 14). It had been previously shown that mortality in similar untreated goats was 100 % for the Senegal isolate (12 out of 12), Welgevonden (5/5), Gardel (5/5), Um Banein (4/4) and Lutale (4/4). The Umpala stock was also highly virulent but was tested in two animals only (both died). The Ball 3 isolate appeared somewhat less pathogenic with a mortality rate in untreated goats of 10 out of 13 (10).

Cultivation

The method of cultivation of *Cowdria* has been described before (12). Briefly, bovine umbilical endothelial (BUE) cells were grown in RPMI 1640 medium. Monolayers were inoculated with *Cowdria* (Senegal stock) and incubated on a slowly rocking platform. *Cowdria* growth medium consisted of Glasgow Minimal Essential Medium (GMEM) supplemented with 2.9 g/l tryptose phosphate broth, penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (1.25 µg/ml), HEPES buffer, L-glutamine

(2 mM) and 10 % newborn calf serum. The growth cycle of *Cowdria* consisted of reticulate bodies (RB) within BUE cells resulting in elementary bodies (EB) which were released into the culture medium (11). *Cowdria* infection of BUE cells was scored as follows : RBs (1+), scanty intracellular colonies, less than 1% of BUE cells infected ; (2+), approximately 10% of the cells infected ; (3+), virtually all cells infected. Score for EBs : (1+), scanty extracellular particles ; (2+), present in large numbers, coinciding with moderate cytopathic effect ; (3+), heavily infected culture supernatant, coinciding with destruction of most BUE cells.

Cultures with 3+EBs were used to passage *Cowdria* onto other BUE cells with an average interval of 10.3 days (range 8-34 days) between passages. BUE culture supernatant heavily infected with elementary bodies (score of 3+) of passage n° 21 (324 days in culture) was centrifuged at 10,000 g for 10 min, washed and resuspended in sucrose-phosphate-glutamate buffer (SPG) (2) and stored at -80°C.

Vaccination

At Utrecht, a total of 39 adult female Tesselaar sheep were inoculated intravenously with attenuated *Cowdria* of passage 21, at a dose of 0.5 ml of culture supernatant, deepfrozen at -80°C in SPG buffer. The animals were monitored by daily temperature records, clinical inspection, as well as collection of blood samples for serology at weekly intervals. All vaccinated animals were challenged by the intravenous route on day 30 post infection with 2 ml of virulent blood stabilates. These had earlier been shown to cause fatal heartwater in control animals. The first group, which consisted of 10 sheep, received an homologous challenge by inoculation of virulent blood stabilate (Cr111) infected with the Senegal stock. The remaining 29 animals were divided into 5 groups of 5 animals and one group consisting of 4 sheep. Each group was challenged with a different *Cowdria* isolate, either Umpala, Lutale, Gardel, Ball 3, Um Banein or Welgevonden, 30 days after the animals had been vaccinated.

In Senegal, 60 local sheep from the northern Sahel zone, where *Amblyomma* ticks and heartwater are rare and sheep have been shown before to be susceptible to the disease (6), were transported to the laboratory in Dakar and maintained free from ticks. Serum was prepared from the animals and tested in the indirect fluorescent antibody test (IFA test). 30 of the animals were then immunized with attenuated EBs of the 21st passage of the Senegal stock, as described above. The animals are presently exposed, since 57 days after immunization, together with the 30 non-immunized controls, to natural infestation by ticks in the coastal Niayes region of Senegal, north of Dakar, where *A.variegatum* and heartwater are common (6).

Immunofluorescence test

BUE cultures infected with *Cowdria* (EB score 3+) were centrifuged at 4 °C for 15 minutes at 15,000 g. Pellets were resuspended in PBS, spotted onto microscope slides, dried and fixed in acetone. The slides were incubated with twofold titrations of sera in PBS starting from 1:80 up to 1:20,480. Positive and negative control sera were also tested. Fluorescein isothiocyanate-labeled rabbit anti-sheep immunoglobulins were used as second antibodies. Fluorescence was observed with an Olympus BH2-RFL microscope.

Monitoring

Serum was prepared for the IFA test prior to immunization and at weekly intervals thereafter. Rectal temperatures were recorded daily and the animals were inspected daily for clinical symptoms. Brain smears of animals that died were examined for clusters of *C. ruminantium* in capillary endothelial cells, after methanol fixation and Giemsa staining.

RESULTS

All sheep vaccinated at Utrecht developed antibodies to *Cowdria* with titres ranging from 640 to at least 5120 as determined by immunofluorescence. Six out of the 39 animals had elevated temperatures for a maximum of 2 days, but no further clinical response to the vaccine was observed.

Challenge of 10 of the sheep (n° 373 to 382) with the virulent homologous Senegal stock, previously shown to be lethal for all non-vaccinated control animals, did not provoke any clinical reaction, demonstrating that these animals were solidly immune (table I).

The other 29 sheep vaccinated at Utrecht with the attenuated material were challenged with heterologous isolates. Reactions varied widely from no clinical reaction at all to fatal cowdriosis, depending on the stock of *Cowdria* used for the challenge (table I). Four out of 5 sheep were fully protected against Umpala, whereas one animal reacted with a transient fever only. Three out of 5 sheep challenged with the Lutale isolate were immune, one was partially immune and the fifth animal required tetracycline treatment to prevent a possibly fatal outcome of the disease. Reactions to Ball 3 and Gardel stocks were similar : 2 out of 5 sheep were immune, whereas the remaining animals were partially immune or required tetracycline[1] treatment. The remaining 4 sheep challenged with the

1. Oxytetracycline (Engemycin®) at 20 mg/kg IM.

TABLE I Clinical reactions of sheep vaccinated with attenuated **Cowdria ruminantium** *(Senegal stock) to challenge with homologous and heterologous* **Cowdria** *isolates.*

Sheep number	Challenge stock	Incubation period (days)	Maximum temp. (°C)	Duration of fever (days)	Time to death (days)	Outcome	IFA titre*
373	Senegal	–	–	–	–	no reaction = immune	≥ 5120
374	Senegal	–	–	–	–	no reaction = immune	≥ 5120
375	Senegal	–	–	–	–	no reaction = immune	≥ 5120
376	Senegal	–	–	–	–	no reaction = immune	2560
377	Senegal	–	–	–	–	no reaction = immune	2560
378	Senegal	–	–	–	–	no reaction = immune	≥ 5120
379	Senegal	–	–	–	–	no reaction = immune	640
380	Senegal	–	–	–	–	no reaction = immune	640
381	Senegal	–	–	–	–	no reaction = immune	640
382	Senegal	–	–	–	–	no reaction = immune	≥ 5120
394	Umpala	17	40.6	2	–	partially immune	≥ 5120
412	Umpala	–	–	–	–	no reaction = immune	≥ 5120
408	Umpala	–	–	–	–	no reaction = immune	2560
396	Umpala	–	–	–	–	no reaction = immune	2560
393	Umpala	–	–	–	–	no reaction = immune	≥ 5120
400	Lutale	2	41.2	5	–	Engemycin treatment	640
386	Lutale	9	40.1	2	–	partially immune	2560
406	Lutale	–	–	–	–	no reaction = immune	≥ 5120
398	Lutale	–	–	–	–	no reaction = immune	≥ 5120
388	Lutale	–	–	–	–	no reaction = immune	≥ 5120
403	Gardel	15	40.7	3	–	Engemycin treatment	2560
401	Gardel	15	41.1	3	–	Engemycin treatment	2560
402	Gardel	16	40.5	6	–	partially immune	1280
409	Gardel	–	–	–	–	no reaction = immune	2560
405	Gardel	–	–	–	–	no reaction = immune	1280
399	Ball 3	13	41.7	4	–	Engemycin treatment	≥ 5120
390	Ball 3	14	40.7	3	–	Engemycin treatment	≥ 5120
397	Ball 3	15	41.0	3	–	partially immune	≥ 5120
391	Ball 3	–	–	–	–	no reaction = immune	≥ 5120
383	Ball 3	–	–	–	–	no reaction = immune	≥ 5120
395	Um Banein	9	40.9	5	14	fatal heartwater**	≥ 5120
411	Um Banein	10	41.6	5	–	Engemycin treatment	≥ 5120
404	Um Banein	11	41.9	4	–	Engemycin treatment	≥ 5120
389	Um Banein	12	41.1	3	–	Engemycin treatment	640
387	Um Banein	12	41.5	2	–	Engemycin treatment	≥ 5120
384	Welgevonden	9	41.7	8	19	fatal heartwater**	≥ 5120
407	Welgevonden	12	41.7	6	18	fatal heartwater**	≥ 5120
410	Welgevonden	12	40.4	1	14	fatal heartwater**	≥ 5120
392	Welgevonden	12	41.6	3	15	fatal heartwater**	640

* *All sheep were negative for* Cowdria *antibodies prior to vaccination ; the IFA titre was determined four weeks after vaccination but prior to challenge inoculation.*
** *Heartwater confirmed by the demonstration of rickettsial inclusion bodies in brain crush smears.*

Um Banein isolate required treatment, after one of them had died. Finally, the attenuated vaccine did not protect at all against challenge with Welgevonden, resulting in death due to cowdriosis of all 4 vaccinated sheep.

Antibody levels after vaccination did not correlate with the level of protection induced.

In Senegal, 13 of the 30 vaccinated sheep had a febrile response after vaccination, and a temporary diarrhoea

was observed. No other clinical signs were noticed and none of the animals was treated. Results of exposure to field challenge will be reported later.

DISCUSSION

In the first report on vaccination with live attenuated *Cowdria ruminantium* the vaccinated animals were challenged with the homologous virulent Senegal stock (12). In this study it is confirmed that a solid protective immunity can be induced in sheep (n = 10) against a lethal challenge with the homologous isolate. In view of possible replacement of current vaccination using virulent blood with in vitro attenuated organisms, it was important to determine reponses to heterologous challenge under laboratory conditions. It was found that responses to heterologous *Cowdria* challenge varied depending on the isolate used. For instance, on the one hand 4 out of 5 sheep were protected against challenge with the Umpala isolate, whereas on the other hand 4 out of 4 sheep died due to challenge with the Welgevonden isolate.

It has been demonstrated previously that antigenic differences between stocks of *Cowdria* play an important role in small ruminants (5, 9, 10). For instance, cross-immunity experiments in goats have shown that 3 out of 5 goats immunized with the virulent Senegal stock died of heartwater after challenge with the Welgevonden isolate (10). Therefore, the fatal outcome of heterologous challenge of sheep with the Welgevonden isolate in the present study could be expected, although immunological differences between the two isolates appear to be much more pronounced in goats than in sheep (10).

Responses of vaccinated sheep to challenge with Ball 3, Lutale, Gardel and Um Banein isolates were heterogenous. Two or three animals in each group were fully protected, whereas the remaining animals were partially immune or required treatment, apart from all 5 sheep challenged with the Um Banein isolate, which all reacted severely. This was surprising in view of the fact that complete cross-protection between this stock and Ball 3, Lutale and Gardel isolates has been reported in goats (10, 14, 15). Finally, 4 out of 5 sheep challenged with the Umpala isolate from Mozambique were protected, indicating a high level of cross-immunity between this isolate and the attenuated *Cowdria* from Senegal.

It can be concluded that antigenic differences are an important factor in the development of improved vaccination methods to prevent cowdriosis. It should however also be stressed that lack of cross-protection between stocks may be more pronounced in small ruminants than in cattle. It is therefore important to determine the extent of heterologous field challenge in cattle vaccinated with attenuated *Cowdria*, in addition to experiments with small ruminants. The attenuated vaccine is currently tested in a field trial using sheep in Senegal, the results of which will be reported elsewhere. Further experiments are underway to determine whether attenuation of other isolates can also be achieved. Finally, it remains to be shown if *Amblyomma* ticks feeding on vaccinated animals will transmit avirulent or virulent rickettsiae.

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JONGEJAN (F.), VOGEL (S.W.), GUEYE (A.), UILENBERG (G.). Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 223-227

Sequential passage of *Cowdria ruminantium* (Senegal isolate) in cultures of bovine umbilical endothelial cells has resulted in loss of virulence without loss of immunogenicity, as previously demonstrated. We have carried out further immunization of 39 Dutch sheep using in vitro attenuated rickettsiae of passage 21 and challenged these animals either with the homologous or with heterologous *Cowdria* stocks. After vaccination several sheep developed elevated rectal temperatures for a maximum of 2 days, but no further clinical response to the vaccine was observed. All sheep developed high titres of antibodies to *Cowdria*. Challenge of 10 sheep with the homologous virulent stock did not provoke any clinical reaction, demonstrating that these animals were solidly immune. Reactions to heterologous challenge varied from virtually no reaction to fatal heartwater depending on the stock of *Cowdria* used. These results are discussed in relation to currently available vaccination methods against cowdriosis. In Senegal 30 susceptible sahelian sheep were immunized with attenuated rickettsiae of passage 21. Hyperthermia was seen in 13, the only other clinical symptom was a temporary diarrhoea. The immunized animals are at present exposed, together with 30 controls, to field challenge in the Niayes, the area where the Senegal isolate was originally isolated.

Key words : Sheep - Heartwater - *Cowdria ruminantium* - Cell growth - Bovine endothelial cell - Immunological technique - Attenuated vaccine - Antibody - Vaccination - Virulence - Hyperthermia.

JONGEJAN (F.), VOGEL (S.W.), GUEYE (A.), UILENBERG (G.). Vacunación contra cowdriosis mediante el uso de organismos de *Cowdria ruminantium* atenuados *in vitro*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 223-227

Se ha demostrado que los pasajes seguidos de *Cowdria ruminantium* (aislamiento de Senegal) en cultivos de células de endotelio umbilical bovino, resulta en la pérdida de virulencia, sin pérdida de inmunogenicidad. Se llevó a cabo la inmunización de 39 ovejas holandesas, mediante el uso de rickettsias atenuadas in vitro, al pasaje 21. Estos animales se probaron con series homólogas o hetérologas de *Cowdria*. Después de la vacunación, varias ovejas presentaron temperaturas rectales elevadas, durante un máximo de dos días, pero no se observaron otros signos clínicos secundarios a la vacuna. Todas las ovejas desarrollaron títulos altos de anticuerpos contra *Cowdria*. Diez (10) ovejas fueron sometidas a una serie homóloga virulenta, sín presencia de reacciones clínicas, lo que demuestra la sólida inmunidad de estos animales. Las reacciones a las series heterólogas variaron de la ausencia de reacción hasta cowdriosis fatal, según el tipo de serie. Estos resultados se discuten en relación a los métodos existentes de vacunación contra la cowdriosis. En Senegal, se inmunizaron 30 ovejas sahelinas susceptibles, con rickettsias atenuadas al pasaje 21. En trece de ellas se observó hipertermia. El otro síntoma clínico presente fue una diarrea pasajera. Los animales inmunizados se encuentran actualmente expuestos, junto con 30 controles, a pruebas de campo en Niayes, zona de origen del aislamiento senegalés.

Palabras claves : Ovino - Cowdriosis - *Cowdria ruminantium* - Cultivo de célula - Célula endotelial bovina - Técnica inmunologica - Vacuna atenuada - Anticuerpo - Vacunación - Virulencia - Hipertermia.